Epimeredinoside A, and the Pharmaceutics of Epimeredinoside A-contained *Epimeredi indica* root extract and its preparatory methods

Field of the Invention

This invention involves in the filed of TCM pharmaceutics, mainly dealing with anti- female menopausal syndrome effective epimeredinoside A, and epimeredinoside A -contained pharmaceutics of *Epimeredi indica* extract and the pharmaceutics' preparatory method.

Background of the Invention

Estrogen and its pharmaceuticss have been applied widely for the treatment of menopausal syndrome for a long time. However, it is hard to be accepted by women for many side effects and adverse reaction, even leading to cancer. Therefore, there is no satisfactory clinic drug at present.

Epimeredi indica (L.) Rothmalex, Guang-Fang-Feng, also named as Fang-Feng-Cao, which is recorded in *The Dictionary of Traditional Medicine*, is the whole plant of *Epimeredi indica* in Labiatae family. And it has been used in the treatment of many disorders such as cold with fever, disgorging, abdominal pain, bones and muscles pain, pyocutaneous disease, eczema, hemorrhoids and so on. It is used in the formula of Guanfang Ganmao Pills recorded in Volume 20 of *Zhong-Yao-Cheng-Fang-Zhi-Ji* (the TCM Pharmaceutics of Patent Formula)published by the Ministry of Public Health of the People's Republic of China, .

New usage of *Epimeredi indica* root has been announced in Chinese Patent No.02110522.7 by the inventor. *Epimeredi indica* root has the effects of ameliorating ovary function and regulating estrogen and progestogen , therefore it can be used to prepare drugs and health care products to treat and prevent many diseases due to the imbalance of estrogen and progestrogen.

Summary of the Invention

The present invention further develops pharmaceutics of *Epimeredi indica* root extract on the basis of Chinese Patent No.02110522.7, about a noval oral pharmaceutics with clear active constituent and its content and stable quality.

The present invention announces all kinds of pharmaceutics related to any oral pharmaceutics, composed of *Epimeredi indica* root extract and pharmaceutical adjuvant. This extract is obtained from extracts of *Epimeredi indica* root after being extracted by water and

concentrated by distillation, containing 0.10% to 1.50% of epimeredinoside A.

Pharmaceutical adjuvants involved in present invention are all common adjuvants in regular pharmaceutics. The oral pharmaceutics are any oral dosage forms widely used in medical area including hard capsule, soft capsule, granule, tablet, oral liquid and so on.

Another technical point announced in the present invention is the preparatory method of the extract and determination method of active constituents in this it.

Preparatory method of *Epimeredi indica* root extracts in the present invention comprise the following steps:

1. Powdering the roots of the *Epimeredi indica*, then add 10 times amount of water to extract for two times,1~2 hours per time. After filtration, it was concentrated as extracta sicca to a density of 1.01 to 1.08(25~30℃), then dried by spray or vacuum. The contents of epimeredinoside A in this extract are 0.10 to 1.50% by HPLC determination.

2. Mix extracts and adjuvants well in proportion to prepare various pharmaceutics conventionally by wet or dry granulation.

Content determination method of Epimeredinoside A in extracts of *Epimeredi indica* root in the present invention comprises the following steps of:

1. Apparatus and Materials:

Instrument: Agilent 1100 HPLC system

Standard: epimeredinoside A

Chemical reagents: methanol, acetonitrile, distilled water and other reagents were HPLC grade

Sample: Extracts of *Epimeredi indica* root (Shanghai Yaogang Biotechnology Ltd.Co.)

2. Chromatographic conditions:

Chromatographic column: Discovery C_{18} (250mm ×4.6 mm, 5μm)

Mobile phase: acetonitrile: water= 27:73

Flow rate: 1.0ml/min Column temperature: room temperature

Detection wavelength: 320nm

Injection volume: 20ul

3. Calibration curve:

① Preparation of standard stock solutions: The standard (4.95 mg) were weighed, dissolved, and diluted with methanol in a 25 ml volumetric flask to obtain standard stock

solutions for the calibration curves.

②The Calibration Curves: The stock solution 0.4, 0.8, 1.2, 1.6, 2.0 ml were weighed, dissolved, and diluted with methanol in 2 ml volumetric flask to obtain standard solutions at the concentration of 39.6 μg/ml, 79.2 μg/ml, 118.8 μg/ml, 158.4 μg/ml, 198 μg/ml respectively. A total of 20 μL of each standard solution was subject to HPLC quantitative analysis. A calibration curve was generated to confirm the linear relationship between the peak area ratio (Y axis) and the concentrations of the standard (X axis) in the test samples. The calibration curves were found to be linear and could be described by the regression equations Y=20.139 X – 154.35, with coefficience of R^2 = 0.9994. The ranges of calibration curves was 0.792 – 3.96 μg, and the retention time of epimeredinoside A was 9.55 min.

4. Sample determination

Preparation of the standard solutions: The standard was accurately weighed, dissolved, and diluted with methanol in a volumetric flask to obtain standard solutions. A total of 20 μL of standard solution was subject to HPLC quantitative analysis and the peak area was recorded. The contents of epimeredinoside A was calculated using the calibration curves accordingly, see Fig 2.

Preparation of the sample solutions: The extracts of *Epimeredi indica* root (176.66 mg) was ccurately weighted, and extracted with by ultrasonication at room temperature for 2 times, then centrifuged. The supernatant were combined and diluted with water in a 10 ml volumetric flask. The solution was filtered through a syringe filter (0.45 μm).

The sample solutions were subjected to HPLC analysis as described above. The content of epimeredinoside A in samples were calculated according to the calibration curves.

Formula for calculation is as follows:

Y=20.139X-154.35

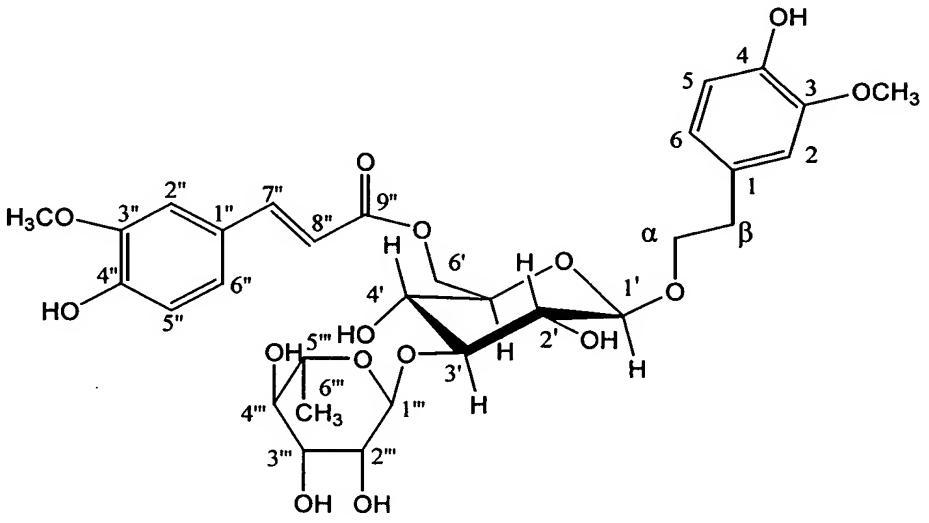
Y : value of peak area

X: value of sample concentration (μg/ml)

The contents of epimeredinoside A in sample is demonstrated as X*10/*amount of sample*100%

Epimeredinoside A used in the present invention is an active compound obtained from extracts of *Epimeredi indica* root through isolation and purification. Extracts of *Epimeredi indica* root was extracted with n-butanol. The soluble extracts were then chromatographed on macroporous

resin and C-18 silicon column, eluted with ethanol gradient, collected and assayed by TLC. The ethanol elute was concentrated for obtaining epimeredinoside A. Figure 2 is its chromatogram of HPLC. Its structure is showed as follows:



Validation of the HPLC methods for determination epimeredinoside A in present invention:

(1) Calibration curve:

① Preparation of standard stock solutions: The standard (4.95 mg) were weighed, dissolved, and diluted with methanol in a 25 ml volumetric flask to obtain standard stock solutions for the calibration curves.

②The Calibration Curves: The stock solution 0.4, 0.8, 1.2, 1.6, 2.0 ml were weighed, dissolved, and diluted with methanol in 2 ml volumetric flask to obtain standard solutions at the concentration of 39.6 μg/ml, 79.2 μg/ml, 118.8 μg/ml, 158.4 μg/ml, 198 μg/ml respectively. A total of 20 μL of each standard solution was subject to HPLC quantitative analysis. A calibration curve was generated to confirm the linear relationship between the peak area ratio (Y axis) and the concentrations of the standard (X axis) in the test samples. The calibration curves were found to be linear and could be described by the regression equations Y=20.139 X – 154.35, with coefficience of R^2 = 0.9994. The ranges of calibration curves was 0.792 – 3.96 μg, and the retention time of epimeredinoside A was 9.55 min.

Peak area

Number	1	2	3	4	5
Sample concentration(μg/ml)	39.6	79.2	118.8	154.4	198
Peak area (mAU)	612.811	1472.17	2234.391	3036.277	3802.776

Calibration of epimeredinoside A is given in figure 1.

(2) Precision

To imbibe standard solution at concentration of 0.198mg/ml for precision study on above HPLC chromatographic condition, then inject above standard solution six times consecutively.

Number	Peak area	X	RSD (%)
1	3802.776	3815.223	0.824
2	3806.568		
3	3879.024		
4	3796.254		
5	3802.456		
6	3804.259		

The results showed that precision of this method is preferable.

(3) Stability

Peak area of standard solution was assayed at 0, 4, 8,12h with injection volume of 20ul per time.

Number	1	2	3	4
Peak area	3785.21	3749.56	3802.54	3855.23
Mean	3798.135			
RSD (%)	1.16			

(4) Reproducibility

Five samples that have the same patch number were prepared for measurement according to criteria on sample assay procedure mentioned above. Peak area of epimeredinoside A in sample solution was assayed with injection volume of 20μl.

Number	1	2	3	4	5
Peak area	522.824	531.245	536.258	522.356	514.252
Mean	525.387				
RSD (%)	1.63				

(5) Recovery

The determined samples were weighed accurately and the standard epimeredinoside A solution were added into the samples accordingly, and the content of epimeredinoside A in samples were determined under the same conditions as described above.

NO.	Sample/μg	Added/μg	Analysis/μg	Recovery	Average	RSD(%)

1	38.643	31.68	68.495	97.400		
2	38.643	31.68	66.455	94.500		
3	38.643	39.6	72.922	93.199	98.292	5.26
4	38.643	39.6	74.8	95.600		
5	38.643	47.52	99.362	102.552		
6	38.643	47.52	91.764	106.500		

The results showed that a sensitive and stability analysis method for the determination of *Epimeredi indica* Root Extract was established.

The invention, pharmaceutics of *Epimeredi indica* Root Extract DO NOT contain any hormone. NO progesterone is needed to be intaken to prevent the side effect after using the drug. It is compatible for the female menopause that the drug has doubtless effect in clinic, stability, controllable and safety. Furthermore, a new approach was provided for the patients which need using estrogen but with contraindication of hormone.

Brief Description of the Drawings

Fig.1: Calibration curve of the epimeredinoside A.

Fig.2: HPLC chromatogram of epimeredinoside A.

Fig.3: HPLC chromatogram of Epimeredi indica Root Extract.

Detailed Description of the Invention

Example 1 Preparation of epimeredinoside A

（1） The dried and powdered root of *Epimeredi indica* was extracted with 10 folder water for 2 hours, filtered. The residue was extracted with 8 folder water for 2 hours again, and filtered. The filter were combined and evaporated under vacuum to afford *Epimeredi indica* Root Extracts.

(2) The 6 kg of *Epimeredi indica* Root Extracts was extracted with 10 folder water for 3 times, and the solvent was evaporated to 600 ml. The residue was extracted with aqua-saturated n-butanol for 3 times (400 ml/ time). The n-butanol solvent was evaporated under vacuum. The extracts of n-butanol was dissolved in water and chromatographied macroporous resin column (AB-8, Nankai Chemistry Factory, Tianjin). The chromatographic column was eluted with a gradient mixtures of 20%, 50% and 95% aqueous ethanol successively. The elutes of 50% ethanol was concentrated and then dissolved with 50% aqueous methanol. The samples of

50% methanol was chromatographed on a RP-C18 silica column , eluted with 50% aqueous methanol to produce epimeredinoside A.

The structure of epimeredinoside A was elucidated by UV, IR, ESI, HRESI, NMR, 2D-NMR (COSY, HMQC, HMBC, NOESY) data. Epimeredinoside A, mp 139~142℃, molecular formula of $C_{13}H_{40}O_{15}$ and the molecular weight 652, was isolated. The ^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) spectral data of Epimeredinoside A ($CDCl_3$) was shown in **Table 1.**

Table 1: ^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) spectral data of Epimeredinoside A ($CDCl_3$)

Ferulic acid	δ C	Δ H	Aglycone	Δ C	δ H
1	127.68		1	132.69	
2	111.66	7.15 (d,2)	2	117.00	6.69 (d,2)
3	150.64		3	147.47	
4	149.36		4	147.33	
5	116.47	6.80 (d,8)	5	112.81	6.65 (d,8)
6	124.27	7.02 (dd,8,2)	6	121.11	6.61 (dd,8,2)
7	147.10	7.62 (d, 16)	α	36.71	2.80 (t,7)
8	115.28	6.39 (d,16)	β	72.31	3.5 –4.2
9	169.07		OCH3	55.40	3.76 (s)
OCH3	55.44	3.86 (s)			
Glucose	**δ C**	**Δ H**	**Rhamnose**	**Δ C**	**δ H**
1	104.39	4.33 (d,8)	1	102.73	5.18 (d,1)
2	75.66	3.5—4.2	2	72.34	3.5 –4.2
3	84.08	3.53 (m)	3	72.25	3.5 –4.2
4	70.54	3.5 – 4.2	4	73.99	3.5 –4.2
5	75.37	3.5 –4.2	5	70.05	3.5 –4.2
6	64.48	4.41 (m)	6	17.88	1.25 (d,6)

Example 2 Preparation and Quantitative Analysis of *Epimeredi indica* Root Extract

A: The dried and powdered root of *Epimeredi indica* was extracted with 10 folder water for was and filtered, the residue was extracted with 8 folder waterfor 2 hours again, filtered. The filters were combined and concentrated under vacuum to obtain the extracts of *Epimeredi indica* Root.

B: Quantitative Analysis

1. Apparatus and Materials

Apparatus: Angilent 1100 HPLC system.

Standard: Epimeredinoside A

Chemical reagents: Methanol, acetonitrile, water and other chemical reagents were HPLC-grade.

Samples: Extracts of *Epimeredi indica* Root (Shanghai Yaogang Biotech Co. Ltd)

2. Chromatographic conditions

Column: Discovery C18 (250*4.6 mm, 5μm)

Mobile phase: Acetonitrile : Water = 27: 73

Flow rate: 1.0 ml/min

Column temperature: Room temperature

Detector wavelength: 320 nm

Injection volume: 20μl

3. Calibration curves

① Preparation of standard stock solutions: The standard (4.95 mg) were weighed, dissolved, and diluted with methanol in a 25 ml volumetric flask to obtain standard stock solutions for the calibration curves.

②The Calibration Curves: The stock solution 0.4, 0.8, 1.2, 1.6, 2.0 ml were weighed, dissolved, and diluted with methanol in 2 ml volumetric flask to obtain standard solutions at the concentration of 39.6 μg/ml, 79.2 μg/ml, 118.8 μg/ml, 158.4 μg/ml, 198 μg/ml respectively. A total of 20 μL of each standard solution was subject to HPLC quantitative analysis. A calibration curve was generated to confirm the linear relationship between the peak area ratio (Y axis) and the concentrations of the standard (X axis) in the test samples. The calibration curves were found to be linear and could be described by the regression equations Y=20.139 X – 154.35, with coefficience of R^2 = 0.9994. The ranges of calibration curves was 0.792 – 3.96 μg, and the retention time of epimeredinoside A was 9.55 min.

4. Samples analysis

Preparation of the standard solutions: The standard was accurately weighed, dissolved, and diluted with methanol in a volumetric flask to obtain standard solutions. A total of 20 μL of standard solution was subject to HPLC quantitative analysis and the peak area was recorded.

The contents of epimeredinoside A was calculated using the calibration curves accordingly, see Fig 2.

Preparation of the sample solutions: The extracts of *Epimeredi indica* root (176.66 mg) was ccurately weighted, and extracted with by ultrasonication at room temperature for 2 times, then centrifuged. The supernatant were combined and diluted with water in a 10 ml volumetric flask. The solution was filtered through a syringe filter (0.45 μm).

The sample solutions were subjected to HPLC analysis as described above, shown in Fig. 3. The content of epimeredinoside A in samples were calculated according to the calibration curves.

Peak area (Y): 383.380,

The concentration X is 26.70 μg/ml according to the regression equations Y=20.139 X – 154.35.

The content of epimeredinoside A in sample was 0.15% by the equation X*10 / Sample Amount*100%.

Example 3 Preparationof the grannule

Formula:

Extracts of *Epimeredi indica* Root	150 g
Lactose	50 g
Stearate Magnesium	2 g

Methods: The extracts of *Epimeredi indica* Root which prepared as described in examole 2 was mixed with lactose and stearate magnesium and then sieved. The granule was obtained by sieving again. The content of epimeredinoside A was 0.17 %.

Example 4

Formula:

Extracts of the *Epimeredi indica* Root	130 g
Lactose	70 g
Stearate Magnesium	1 g

Methods: The extracts of *Epimeredi indica* Root which prepared as described in example 2 was mixed with lactose and stearate magnesium and then sieved. The granule was obtained by sieving again. The content of

epimeredinoside A was 0.13 %.

Example 5 Preparation of the Capsule

Formula:

Extracts *Epimeredi indica* Root	110 g
Lactose	90 g
Stearate Magnesium	1 g

Methods: The extracts of *Epimeredi indica* Root which prepared as described in example 2 was mixed with lactose and stearate magnesium and then sieved. The grain was sieved again. And the capsules were filled with the fine grain. The content of epimeredinoside A was 0.27 %.

Example 6 Preparation of the Tablet

Formula:

The extracts of *Epimeredi indica* Root	230 g
Cellulose, Microcrystalline	20 g
Carboxymethyl starch sodium	3 g
Polyvinylpyrrolidone	1 g
Pulvis Talci	1 g
Stearate Magnesium	1 g

Methods: The Microcrystallined Cellulose, Carboxymethyl starch sodium and other materials were mixed in mortar, the extracts of *Epimeredi indica* Root which prepared as described in example 2 was added. The powder was sharped in muller. The fine powder was grannuled, dried and Stearate Magnesium added. The grannule was tableted and coated. The content of epimeredinoside A was 0.23 %.

Example 7 Preparation of the Tablet

Formula:

The extracts of *Epimeredi indica* Root	300g
Cellulose, Microcrystalline	26g
Carboxymethyl starch sodium	2.8g
Polyvinylpyrrolidone	2.8g

Pulvis Talci	2.8g
Stearate Magnesium	1g

Preparation was carried out according to the method mentioned in example 6. The concentration of epimeredinoside A is 0.22%.